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M. R. Bakst

USDA-ARS, murray.bakst@outlook.com

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# PHYSIOLOGY AND ENDOCRINOLOGY SYMPOSIUM: Role of the oviduct in maintaining sustained fertility in hens<sup>1</sup>

M. R. Bakst<sup>2</sup>

Animal Biosciences and Biotechnology Laboratory, ARS-USDA, Beltsville, MD 20705

**ABSTRACT:** In poultry, sperm transferred by natural mating or AI into the distal end of the vagina immediately begin their ascent to the uterovaginal junction (UVJ) at the anterior end of the vagina. However, due to an intense selection process in the vagina, less than 1% of the sperm transferred actually reach the UVJ. Those sperm that do reach the UVJ enter numerous tubular invaginations of the surface epithelium of the vagina located in the UVJ mucosa, collectively referred to as the sperm-storage tubules (SST). Sperm residing in the SST lumen are capable of surviving up to several weeks while retaining their fertilizing capacity. Resident sperm are released gradually from the SST while the hen is in egg production, ascend to the site of fertilization, and interact with the next ovulated ovum. In this manner, given the absence of an estrus to synchronize ovulation with copulation, poultry are ensured a population of sperm at the site of fertilization

around ovulation. Over the past decade, several new and diverse observations have been published addressing the microanatomy of the UVJ and SST, and the cellular and molecular mechanisms orchestrating oviductal sperm selection and storage. These include the role of sperm mobility in selection and transport, SST numbers in different poultry species and lines of high and low fertility, roles of the immune system and possibly neuroendocrine-like cells in the vagina in sperm selection and storage, and the roles of aquaporins and a fluid exchange mechanisms contributing to sperm release from the SST. The objective of this paper is to review and integrate these observations into a comprehensive understanding of the cellular and molecular events influencing the fate of sperm in the oviduct of the hen, particularly with regard to oviductal sperm selection and storage.

**Key words:** avian, oviductal poultry, sperm storage, sperm storage tubule

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## INTRODUCTION

The biology of reproduction is vastly different between birds and mammals. In the absence of a mammalian-type estrous cycle for the synchronization of copulation with ovulation, birds rely on oviductal sperm storage. In the domestic and the nondomestic birds examined, the surface epithelium lining the anterior 2 cm of the vagina, referred to as the uterovaginal junction (UVJ), is modified to form numerous tubular invaginations referred to collectively as the sperm-storage tubules (SST). Shortly before and during egg production,

sperm residing in the SST will, upon release from the SST, ascend the oviduct to the site of fertilization in the infundibulum. Here sperm interact with a near-daily succession of ovulated ova over days to several weeks, depending on the species. For more detailed reviews of the events leading up to fertilization, see Bakst et al. (1994), Wishart and Horrocks (2000), Stepinska and Bakst (2007) for domestic birds, as well as Birkhead and Moller (1992) and Birkhead and Brillard (2007) for nondomestic birds.

A better understanding of the fundamental cellular and molecular mechanisms regulating oviductal sperm selection, transport, and storage would have a profound effect on the breeding sector of the commercial poultry industry. This information could lead to 1) more innovative approaches to the development of semen extenders that maintain sperm viability at ambient temperature for greater than 24 h, 2) the use of gene markers for the selection of the most fecund breeders, 3) providing sound scientific information rather than empirical observations to poultry flock managers when confronting fertility problems, and 4) implementation of novel ap-

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<sup>2</sup>Corresponding author: murray.bakst@ars.usda.gov

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proaches to poultry management, ultimately increasing the ratio of breeder females per male.

Since the mid 1990s, several intriguing observations regarding the fate of sperm in the domestic bird oviduct have been published that resulted in both the re-evaluation of former and the introduction of new concepts in the understanding of reproduction in birds. The objectives of this paper are to review these new observations and then integrate them into a more unified understanding of oviductal-sperm interactions and sustained fertility in birds in general and poultry in particular.

## ANATOMY AND HISTOLOGY OF THE UTERUS AND VAGINA

The structure and function of the avian oviduct has been extensively described (see Introduction). Surprisingly, the gross anatomy of the avian vagina, which is now understood to be quite complex, was not described in detail until recently. In describing the duck vagina, Brennan et al. (2007) observed a spiral-shaped tube that was further characterized by blind pouches stemming from its distal half. When considering the waterfowl penis is corkscrewed with an opposite orientation to the spirals in the vagina (Brennan et al., 2010), the authors concluded that the anatomical incongruity between the phallus and vagina would be used by the female to impede the penetration of the penis during attempts at forced copulation.

Although Bakst (1998) alluded to the turkey vagina as being tightly coiled and enveloped by connective tissue, it was the work of Brennan et al. (2007) that motivated a more detailed anatomical examination of the turkey vagina and uterus. Bakst and Akuffo (2009) fixed the turkey vagina and uterus *in toto*, with and without an egg mass in the uterus. The connective tissue binding the vagina and uterus was then removed and revealed a spiral configuration regardless of whether or not an egg mass was present in the uterus (Figure 1A). Without an egg mass in the uterus, the UVJ mucosal folds containing SST clearly did not extend into the uterus and were contiguous with the vaginal mucosa (Figure 1B). Alternatively, when an egg mass was present in the uterus, the UVJ folds containing SST were contiguous with the uterine mucosa and clearly within the uterus pouch (Figure 1C). This anatomical configuration would indicate that sperm exiting the SST are subjected to uterine fluids known to stimulate chicken sperm motility *in vitro* (Brillard et al., 1987).

The cellular and molecular mechanisms responsible for the morphogenesis of the SST are not known. The anatomical differentiation of the SST has briefly been described by Bakst (1992) in 30-wk-old turkey hens before the onset of photostimulation to bring hens into egg production. Both elongated SST and bud-like surface invaginations, presumptive SST, were observed (Figure 1D). A more detailed description of SST morphogenesis in Japanese quail before and during the period

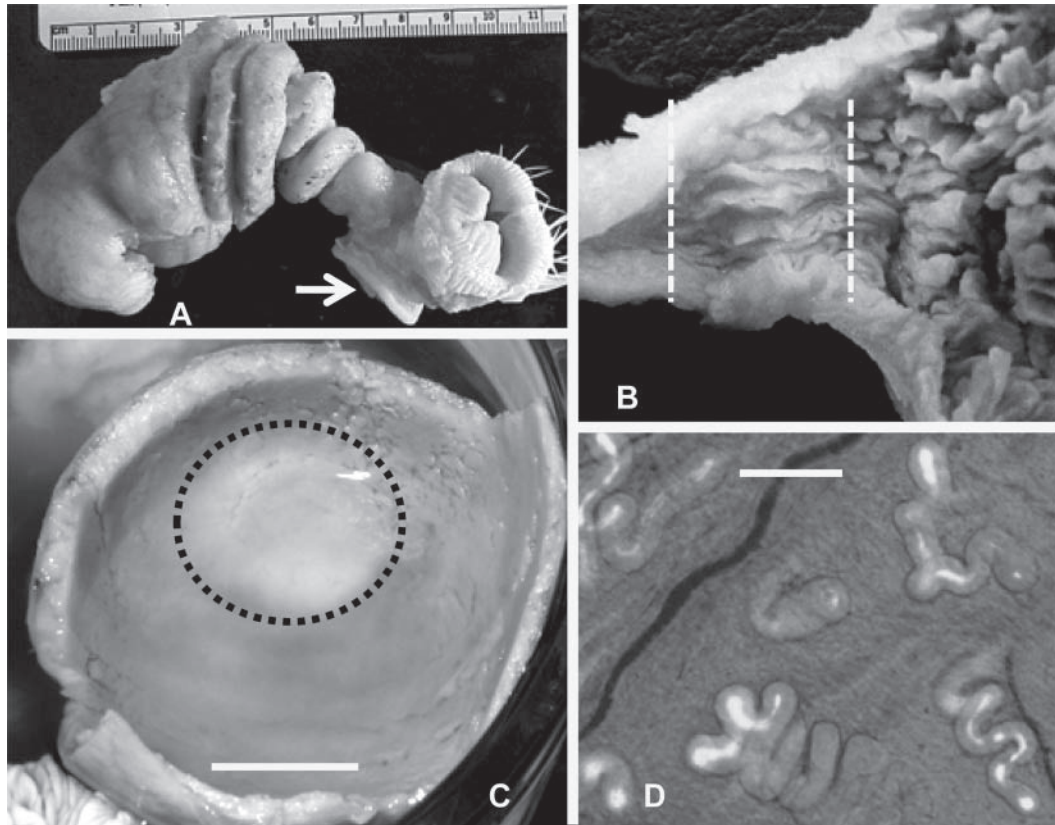
corresponding to ovarian maturation was described by Holm and Ridderstråle (2002). At 28 d of age, the time coinciding with the onset of tubular gland formation in the magnum, low columnar cells were observed at the base of the folds at the UVJ. Within 10 d, these cells had differentiated into bud-like projections and then tubular structures consisting of nonciliated columnar cells, the presumptive SST. Females housed with males possessed sperm in their SST before the first ovulation (approximately 42 d of age). This confirmed earlier observations of sperm in the SST of turkey hens inseminated artificially before the onset of photostimulation and possessing a juvenile oviduct (Bakst, 1988, 1992).

The cell signaling pathways controlling SST differentiation and proliferation during the maturation of the oviduct before the onset of egg production remain unknown. Given the similarity between the cellular organization of luminal mucosae of the intestine and oviduct, one could assume that stromal trophic factors regulate the differentiation and proliferation of the SST in a manner similar to that suggested for the intestine by Simmons et al. (1999). These authors suggested that intestinal crypt cell proliferation and renewal of the cells forming the luminal epithelium were influenced by IGF originating from the subepithelial stroma cells. If such interactions are found to contribute to SST morphogenesis, we then may be able to explain why there exists intra- and interline variation in SST numbers (Bakst et al., 2010).

The total number of SST in the UVJ varies between species (Birkhead and Moller, 1992). More recently, Bakst et al. (2010) calculated the total numbers of SST in 4 strains of broilers of differing fertility and one commercial strain of Large White turkeys. Unlike the numbers of SST for the chicken (13,533) and turkey (20,000) reported by Birkhead and Moller (1992), Bakst et al. (2010) observed that the broiler lines averaged 4,900 SST per hen and turkeys averaged 30,600 SST per hen. Furthermore, Bakst et al. (2010) observed no statistical differences in the SST numbers among the 4 strains of broilers. From these data, Bakst et al. (2010) proposed the following: 1) the longer duration of fertility in turkeys compared with broilers is due, in part, to a greater number SST and a slower daily release of sperm; 2) in a commercial hen flock, variation in fertility is not associated with SST numbers. In contrast, when selected solely for high and low fertility, the number of SST in the high fertility line of hens is significantly greater than the low fertility line of hens (Brillard et al., 1998); and 3) factors other than SST numbers play a role in sustained fertility in commercial strains of broilers and turkeys.

## VAGINA: SPERM SELECTION, TRANSPORT, AND STORAGE

Within the 30 min after the transfer of semen into the vagina, 84% of sperm flow back out of the vagina (Howarth, 1971), most often embedded in a plug of mu-



**Figure 1.** Panel A: the turkey uterus and vagina without an egg mass (excised as 1 segment, fixed, and partially dissected free of the connective tissue capsule enveloping their respective folds). With the ruler used as a guide, the uterus extends from 0 to 5 cm, and the vagina from 6 to 8 cm. The densely coiled uterovaginal junction (UVJ) is located between 5 and 6 cm. The arrow highlights the distal end of the coprodeum that joins, along with the vagina, with the urodeum, the central compartment of the cloaca. The dorsal lip (identified by the pin-feathers) and the ventral lip of the cloaca are also observed. Removal of the enveloping connective tissue reveals the corkscrew-shaped vagina and the larger diameter uterus characterized by deep circumferential folds. Panel B: a fixed specimen identical to that in panel A, with no egg mass, was cut along its longitudinal axis and pinned back to reveal the luminal mucosal folds of the UVJ and uterus. The UVJ folds (between the 2 lines) are narrow and contiguous with the vagina folds. In contrast to the vagina, the uterine folds are more voluminous and the longitudinal orientation is absent due to the deep transverse folding (panel B) of the uterine wall. The distance between the 2 vertical lines is 14 mm. Panel C: a turkey uterus and vagina excised as 1 segment, fixed, and the uterus cut transversely (egg mass was removed after fixation) to visualize the anatomical position of the UVJ. The UVJ folds (circled) are now clearly contiguous with the uterine mucosa. The presence of sperm-storage tubules were confirmed microscopically in the UVJ folds. Bar = 15 mm. Panel D: unfixed squash preparation of a single UVJ fold (turkey; ciliated surface of mucosa is against the slide) containing sperm-storage tubules (SST) with varying numbers of sperm. The luminal sperm, which are fluorescing intensely, were stained with a nuclear fluorescent dye before insemination. Elongated, pleomorphic SST are observed surrounding a shorter, bud-like SST. Bar = 120  $\mu$ m.

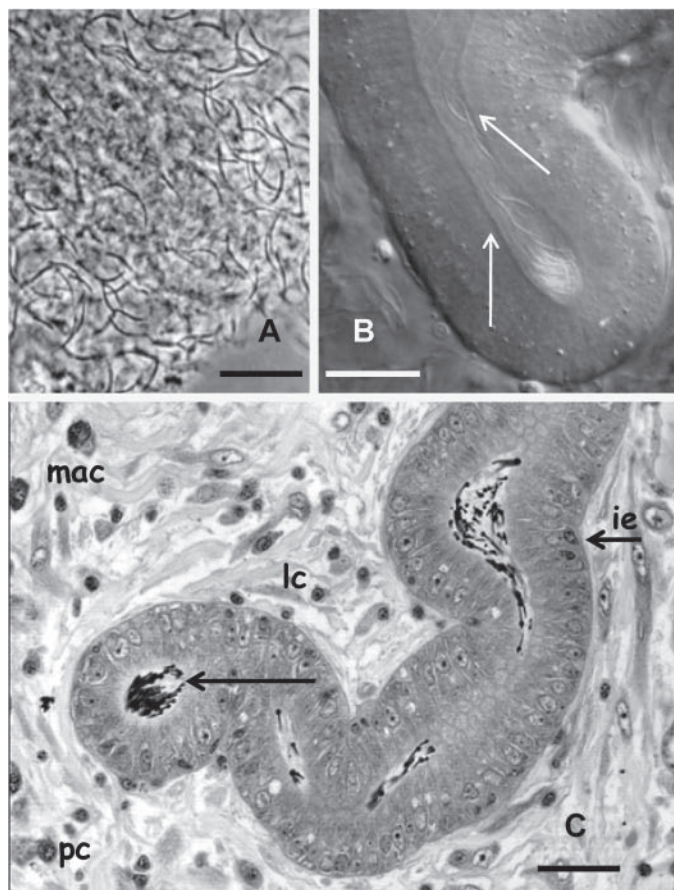
cus (Figure 2A; J. P. Brillard, INRA, Nouzilly, France, personal communication). The remaining sperm are transported in an adovarian direction by a combination of their intrinsic mobility (i.e., capacity to move through a viscous medium; Froman et al., 2006) and the sperm transport mechanisms of the vagina that include smooth muscle activity and the activity of the ciliated cells lining the luminal mucosal surfaces of the vagina.

The abovarian transport of the egg mass through the infundibulum, magnum, and isthmus is due to peristaltic activity initiated by local distention of the oviductal smooth muscle layer (Arjamaa and Talo, 1983). Bakst et al. (1994) speculated that intrinsic sperm motility coupled with a fluid transport mechanism in the troughs between tightly apposed mucosal folds were responsible for rapid sperm transport to the UVJ at the distal end of the vagina. This was based on observations that sperm transferred to the distal end of the vagina of an excised turkey oviduct were observed in

the infundibulum (i.e., a distance of about 80 cm) in less than 10 min (M. R. Bakst, unpublished results).

The cellular and molecular basis of sperm mobility and its role in oviductal sperm storage and transport was recently reviewed by Froman et al. (2011) and will not be addressed in detail in this review. Froman et al. (2011) developed a compelling argument for sperm mobility as being the dominant factor in sperm selection within the vagina. Interestingly, Denk et al. (2005) suggested that the swimming speed and motility of mallard sperm figured more prominently in paternity than postcopulatory sperm selection by the female. Notwithstanding the role of sperm mobility and motility, other factors do influence the numbers of sperm that reach the SST. If vaginal insemination is 2 h before or 2 h after oviposition, oviductal sperm transport is altered and sperm filling of the SST is reduced (Birkhead et al., 1996). We also know that the efficiency of sperm transport in the turkey vagina, as measured by the percentage of SST that are filled, partially filled, or





**Figure 2.** Panel A: a mucus plug containing chicken sperm (heads are slightly curved and filiform in shape) recovered from the cloaca within 1 h of semen transfer, thus accounting, in part, for the significant decline in sperm numbers ascending to the uterovaginal junction. The micrograph was provided by J. P. Brillard (INRA, Nouzilly, France). Bar = 30  $\mu$ m. Panel B: a similar preparation as described in Figure 1D except the distal portion of a single sperm-storage tubule (SST) with luminal sperm is observed. The SST epithelium is nonsecretory, nonciliated, and columnar. The arrows indicate the direction of the fluid flow through the SST. In such a preparation, the tails of the closely aligned sperm move slowly in synchrony. Bar = 40  $\mu$ m. Panel C: this histological section of a single SST with luminal sperm (arrow in SST) also reveals the surrounding loose connective tissue containing several different immune cells and is reminiscent of the intestinal mucosa. Immune cell types observed include macrophages (mac), plasma cells (pc), and lymphocytes (lc). An intraepithelial lymphocyte (ie) is also observed at the base of the SST epithelium. Bar = 20  $\mu$ m.

empty after AI, is most efficient before the onset of egg production. Obviously there are mechanisms (i.e., neuromuscular, cellular, endocrine, or the presence of an egg-mass in the oviduct, or all of the above) that affect sperm selection and transport after the onset of lay.

Differences in luminal pH may influence sperm mobility. Bakst (1980) observed significant differences in the pH of the mid-vaginal mucosa of the broiler that ranged from pH 7.15 within 20 min postoviposition (PO) to pH 7.51 at 8 to 12 h PO. In the manually everted turkey vaginal mucosa (i.e., the distal 1 to 2 cm of the vagina), there are significant pH differences that range from pH 6.95 at (8 to 12 h and 18 to 22 h PO) to pH 7.30 within 10 min after oviposition. These varia-

tions in pH may modulate the mobility of sperm after semen transfer. The localization of carbonic anhydrase in UVJ, SST, and vaginal epithelia also indicates a role for pH in the modulation of sperm motility, possibly with a greater pH augmenting sperm motility in the vagina and a decreased pH depressing sperm motility in the SST lumen (Holm et al., 1996).

There is evidence throughout the animal kingdom that the female is able to exert some influence on which male will fertilize her ova (see Eberhard, 1996). This is not only possible through female mate choice, but also in the postcopulation selection of sperm in the reproductive tract of the female. The latter is referred to as cryptic female choice (Eberhard, 1996). Based on what is known about birds, one would assume that cryptic female choice is most likely to be observed in the vagina, although there may be further sperm selection after release from the SST (Birkhead and Brillard, 2007). A local signal, possibly initiated by a component in seminal plasma or sperm, coupled with a response by the vaginal epithelial cells, may trigger a cascade of events that favor the adovarian transport of these sperm. Sperm signaling may be associated with the sperm plasmalemma glycoproteins, or lack of them (Bakst et al., 1994; Wishart and Horrocks, 2000; Peláez and Long, 2008).

Rzas et al. (1991) first demonstrated the presence of serotonin in the chicken oviduct. More recently, serotonin-positive nonneuronal endocrine-like cells have been localized in turkey vaginal and UVJ epithelia, but not the SST epithelia (Bakst and Akuffo, 2008). Similar cells, known as enterochromaffin cells, are observed in the gut epithelium of other species and appear to regulate a local peristaltic reflex (Olsson and Holmgren, 2001). We are currently examining the possibility that the serotonin-positive cells in the vaginal epithelium of the hen may exert a local impact on sperm motility and transport to the SST. In addition to augmenting localized peristaltic activity in the gastrointestinal tract of the chicken (Kitazawa et al., 2006), serotonin has been shown to stimulate both cilia and sperm beat frequency in a variety of species (Stephens and Prior, 1992). Interestingly, using a computer-assisted sperm motility analysis system, serotonin (at  $10^{-4}$  M but not at  $10^{-6}$  M) statistically increased turkey sperm curvilinear velocity and tail beat frequency (M. R. Bakst, unpublished results). Thus, in the context of cryptic female choice, serotonin-containing cells in the vagina and UVJ, but not in SST epithelia, may augment local sperm motility, vaginal cilia beat frequency, and smooth muscle activity facilitating sperm transport to the SST (Bakst and Akuffo, 2008).

Although the question of how sperm survive within the SST for prolonged periods of time has yet to be definitively explained, it is assumed that resident sperm metabolize endogenous fatty acids (Froman et al., 2011) or other lipids derived from the apical microvilli of the SST epithelium (Bakst et al., 1994). Liposome-like vesicles appear to pinch off the microvillar tips of the SST

epithelial cells and appear to interact with the luminal sperm. This region of the SST epithelium is alkaline phosphatase (AP)-positive (Bakst and Akuffo, 2007) and corresponds to the localization of AP in the rat intestinal luminal epithelium (Narisawa et al., 2003). These authors suggested that AP may function in the transfer of lipid across the enterocyte brush border, and it is speculated that AP may have a similar role in the SST epithelium (Bakst and Akuffo, 2007).

The mechanism(s) of sperm release from the SST has been the subject of speculation for years (see Bakst et al., 1994 for review). More recently, it has been suggested that sperm release from the SST may be a neural-mediated mechanism that initiates contraction of the actin-rich band in the apical cytoplasm of the SST epithelium (Freedman et al., 2001), thus expelling sperm from the SST lumen. Alternatively, Froman et al. (2011) suggested that the sperm residing in the SST lumen are subjected to a fluid current moving toward the SST orifice (Figure 2B). Sperm will remain in the SST lumen as long as their swimming velocity is greater than that of the flow rate of the luminal fluid. Sperm release from the SST would take place when, possibly as a result of waning ATP, sperm motility decreases and they are carried out of the SST with the luminal fluids. The localization of aquaporin-3 in the apical region of the SST epithelium (Zaniboni and Bakst, 2004) would support the suggestion that there is a transfer of fluids from the SST epithelium to the SST lumen.

## IMMUNOLOGICAL ASPECTS OF SPERM SELECTION AND STORAGE

Bakst et al. (1994) provided a comprehensive review of possible roles of the immune system of the oviduct on sperm selection and storage. They noted that there were conflicting observations regarding the role, if any, on sperm antibodies and the decline in hen fertility. Since then, Robertson et al. (2000) demonstrated that chicken and turkey sperm must possess the proper array of plasmalemma-associated proteins and glycoproteins, each with their respective saccharide groups to reach the SST and also to interact with the ovum at the time of fertilization. Furthermore, Steele and Wishart (1992) observed vaginally inseminated sperm-bound immunoglobulin (IgA or IgG) and that 84% of those sperm were dead. Of the remaining viable sperm recovered, 7% bound immunoglobulins but only sperm devoid of immunoglobulins were observed in the SST. It is unlikely that the antibodies binding to sperm were sperm-specific antibodies because immunoglobulins were also observed associated with sperm recovered from virgin hens.

The vaginal insemination of heterologous semen into chickens resulted in few sperm reaching the SST, presumably due to the absence of the specific sperm surface glycoprotein array compatible with the oviductal sperm selection mechanism of the hen. However, when added to explants of UVJ folds, heterologous sperm en-

tered the SST, indicating that sperm selection process is orchestrated by the vagina and not the SST (Wishart and Horrocks, 2000).

In the past decade, there has been a resurgence of studies addressing the role of the immune system in the oviduct of the hen with respect to reproductive function (see review by Das et al., 2008). Classes of immunocompetent cells (i.e., macrophages, antigen-presenting cells expressing MHC class II, CD4<sup>+</sup> and CD8<sup>+</sup> T cells, premature B cells, and plasma cells) and cell products associated with both acquired (Zheng et al., 1998; Zheng and Yoshimura, 1999) and innate immunity (i.e., avian  $\beta$ -defensins; Abdel-Mageed et al., 2008) are expressed within the oviductal mucosa, particularly in the vagina (Figure 2C). The vaginal orifice, as well as the coprodeum (i.e., the anterior compartment of the cloaca and extension of the large intestine), communicates directly with the urodeum, the central compartment of the cloaca. Therefore, it is not surprising that the immune system of the vaginal mucosa is highly differentiated (Bakst and Akuffo, 2009) and histologically reminiscent of the gut-associated lymphoid tissue described for birds (Befus et al., 1980).

The role of estrogens in the cytodifferentiation of the luminal and subluminal epithelia of the oviduct has been established for many years (Berg et al., 2001). Of interest is that the numbers of immunocompetent cells associated with acquired immunity are greater in laying than nonlaying hens and the observation that this has also been associated with increased concentrations of estrogen (Zheng et al., 1998). In their review, Das et al. (2008) indicated that the storage of sperm in the SST necessitates an immunosuppression of sperm antigenicity. Using a low fertility line of hens subjected to repeated inseminations, Das et al. (2005b) observed swollen SST lacking resident sperm and lymphocyte infiltration of the SST. In addition, these authors also noted that the mucosa surrounding the SST possessed increased numbers of antigen-presenting cells expressing MHC class II, CD4<sup>+</sup>, and CD8<sup>+</sup> T phenotypes (Das et al., 2005a). Concurrent with the increased numbers of these immunocompetent cells and the abnormal appearance of the SST was a decrease in the abundance of mRNA for estrogen receptor- $\alpha$  (Das et al., 2006b). Their observations prompted the suggestion that because SST structure and function is estrogen dependent, the decreased mRNA expression of estrogen receptor- $\alpha$ , coupled with the increased numbers of immunocompetent cells in and around the SST, may be directly related to the absence of significant sperm storage within the SST in low fertility hens. These authors also observed that transforming growth factor- $\beta$  (TGF- $\beta$ ) and their receptors (T $\beta$ R) increased in the UVJ when sperm reside in the SST (Das et al., 2006a). Given the immunosuppressive properties of TGF- $\beta$ , and that lymphocytes in the UVJ mucosa possess T $\beta$ R, Das et al. (2006a) suggested that TGF- $\beta$  may suppress immune responses to resident sperm in the SST by UVJ lymphocytes. Das et al. (2008) concluded that this suppression of UVJ

lymphocytes by TGF- $\beta$  may contribute to successful sperm storage in the SST.

## SUMMARY AND CONCLUSIONS

Collating the observations discussed previously into a more comprehensive model of the fate of sperm after transfer to the vagina, the following is suggested: 1) after semen transfer, the majority of sperm are rejected by the vagina and the remaining, high-mobility sperm begin transport to the UVJ; 2) these more fit sperm may be subjected to other sperm selection mechanisms, but the single dominant phenotypic trait affecting transit to the UVJ is sperm mobility; 3) successful sperm storage in the SST is dependent on the establishment of an immuno-privileged status for sperm residing in the SST and this may be estrogen- and TGF- $\beta$ -dependent; 4) sperm residing in the SST subsist on lipid derived from the SST epithelium; 5) sperm residence within the SST is dependent on sperm motility exceeding the velocity of the luminal fluid flow exiting the SST orifice; 6) sperm are transported out of the SST when mitochondrial ATP begins to deplete, motility wanes, and the SST luminal fluid velocity exceeds that of the sperm; 7) sperm released from the SST are exposed to calcium-rich uterine fluids, activated, and ascend to the infundibulum; 8) differences in sustained fertility in different lines of commercial broilers are not a function of SST numbers; and 9) the longer fertile period of turkeys compared with broilers is due to turkeys possessing 5 times as many SST as broilers.

To conclude, sustained fertility in the hen is a complex series of temporal and spatial events that ultimately result in a relatively small number of highly selected sperm at the site of fertilization at the time of ovulation. As sperm numbers in the infundibulum decrease, either due to low sperm numbers in the SST, impaired sperm transport and selection by the vagina, or the inability of the SST to store sperm, fertilization rates will fall. Although AI technology has not progressed significantly over the past 2 decades, we now have the capability to select males producing sperm with greater mobility, the most significant phenotypic trait associated with sperm fecundity. This one advance may eventually contribute to greater fertility and the possibility of longer intervals between successive inseminations.

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